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Specificity of Mutations Induced by the Food-associated Heterocyclic Amine 2-Amino-1-methyl-6-phenylimidazo-[4,5-*b*]-pyridine in Colon Cancer Cell Lines Defective in Mismatch Repair

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ABSTRACT

Recently, we have shown a hypermutable response to the food-associated heterocyclic amine 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]-pyridine (PhIP) in human cells defective in mismatch repair (MMR). These findings suggest that exogenous compounds such as PhIP may play an important role in the generation of tumors in MMR-defective individuals. The specificity of mutations induced by PhIP exposure at the endogenous *HPRT* locus was determined in cell lines defective in MMR to better understand the mutagenic effects of PhIP in MMR-defective individuals and to gain insight into the molecular mechanism of carcinogenesis induced by PhIP. Eighty-six induced *HPRT* mutants from two different cell lines were isolated and sequenced after exposure to 10 μM PhIP. Nineteen (22%) of these mutants contained G:C to T:A transversion mutations, consistent with the promutagenic adduct of PhIP at the C8 position of guanine miscoding with adenine. This level of PhIP-induced G:C to T:A transversions was ~4.5-fold higher than spontaneous G:C to T:A frequencies. Additionally, a hotspot for mutation was observed in a run of six guanines in *HPRT* exon 3, where a total of 23 (27%) of all PhIP-induced mutations occurred. These mutations consisted of transversions, transitions, and frameshift mutations. The increase in mutant frequency at this run of guanines corresponded to a 24-fold elevation above the spontaneous frequency in one cell line and a 3.3-fold increase in the other. These data suggest that PhIP may increase the risk of human carcinogenesis mediated by MMR by increasing mutations at runs of guanine residues. PhIP may thereby promote tumorigenesis by mutating growth-regulating genes that contain runs of guanines in their coding sequences, such as *BAX*, the insulin-like growth factor II receptor *IGFIIR*, and even the mismatch repair gene *hMSH6*.

INTRODUCTION

MMR[2] deficiencies and the associated loss of genomic stability have been well documented in human cancers in recent years (reviewed in Refs. 1–4). First implicated in hereditary nonpolyposis colorectal cancer, MMR deficiencies have since been found in a variety of hereditary and sporadic human cancers, illustrating the crucial role MMR proteins play as tumor suppressors. Significantly elevated levels of mutation are observed spontaneously in MMR-defective cells, at both endogenous loci and at microsatellite sequences (5, 6). This elevated mutational burden imposed by the loss of MMR may be responsible for the elevated risk of tumors in MMR-compromised individuals.

Resistance to the cytotoxic effects of various DNA-damaging agents, including alkylating agents and chemotherapeutic agents, has been demonstrated in cell lines defective in MMR (7–10). This increased viability is associated with induced hypermutability (9–11). These observations have practical implications for human carcinogenesis: cells defective in MMR are more resistant to the cytotoxic effects of normally toxic compounds, resulting in selection for unstable cells. By increasing mutations associated with exposure, there is a higher probability of mutating essential growth control genes that may promote tumorigenesis. It seems logical that there are compounds relevant to daily human exposure that will produce a similar cellular response (*e.g.*, increased resistance and hypermutability) in MMR-defective cells. Exposure to such compounds may have profound effects on the risk of colorectal tumorigenesis in hereditary nonpolyposis colorectal cancer.

Logical candidates relevant to human exposure are HAs, common food mutagens/carcinogens postulated as important etiological agents in human carcinogenesis (reviewed in Refs. 12 and 13). HAs are common pyrolysis products found in cooked beef, fish, and chicken and have been implicated in human colorectal carcinogenesis (12, 13). Recently, we reported an increase in resistance to cytotoxicity and in induced mutability from exposure to the food-associated heterocyclic amine, PhIP, in MMR-defective cells relative to MMR-proficient cells (14). Considering that both PhIP and MMR are linked to colorectal carcinogenesis, it may be that PhIP exposure in cells defective in MMR may play an important role in human colorectal tumorigenesis.

To evaluate the mutagenic effects of PhIP in MMR-defective individuals and ultimately the potential carcinogenic effects, the specificity of mutations induced by PhIP exposure at the endogenous *HPRT* locus was determined in cell lines defective in MMR. A total of 86 induced *HPRT* mutants from the DLD-1 and HCT116 cell lines were isolated and sequenced after exposure to 10 μM PhIP. The PhIP-induced mutational spectra determined in these cell lines were compared with each other and with the spontaneous background mutations observed in each cell line. The results are discussed in regard to a potential synergy between the molecular mechanisms of tumorigenesis for PhIP and MMR.

MATERIALS AND METHODS

Cell Lines. Human colon cancer cell lines DLD-1 and HCT116 were obtained from the American Type Culture Collection (Manassas, VA). The molecular defects in specific *MMR* genes in DLD-1 and HCT116 have been defined previously; the DLD-1 cell line is mutant in the *hMSH6* gene, (15), and HCT116 cells are deficient in *hMLH1* (16). Cell lines were grown in DMEM/Ham's F-12 (1:1) + 10% dialyzed fetal bovine serum (HyClone, Logan, UT).

PhIP Treatment. PhIP treatment was performed as described previously (14). PhIP (Toronto Research Chemicals, Toronto, Canada) was resuspended at 5 mg/ml in DMSO (Sigma, St. Louis, MO) just prior to use. PhIP exposure was in normal medium [DMEM/F-12 (1:1) + 10% fetal bovine serum] in the presence of mouse liver S9 induced with phenobarbital and benzoflavone (Moltox, Boone, NC). Approximately 500 μg of S9 protein was added per ml of media (15 μl of S9 per ml). Metabolic enzyme cofactors NADP (Boehringer Mannheim, Indianapolis, IN) and *dl*-isocitric acid (Sigma) were also added for a final concentration of 1 and 5.8 mM, respectively. Cells were exposed to PhIP for 4 h at 37°C.

Isolation of Independent 6-TG-resistant Clones. Preexisting *HPRT* mutants were eliminated from cell cultures by subculturing in HAT medium [100 μM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine (Sigma)]. HAT-cleansed cultures were plated at a density of 5×10^5 cells per 10-cm dish in

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[2] The abbreviations used are: MMR, mismatch repair; HA, heterocyclic amine; PhIP, 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]-pyridine; *HPRT*, hypoxanthine-guanine phosphoribosyl transferase; 6-TG, 6-thioguanine; dG-C8-PhIP, *N*-(deoxyguanosin-8-yl)-PhIP; AAF, *N*-2-acetylaminofluorene; dG-C8-AAF, *N*-(deoxyguanosin-8-yl)-AAF.

nonselective medium. Twenty-four independent dishes were used for treatment with PhIP. Twenty-four h after plating, cells were exposed to 10 μM PhIP as described above. Cells were maintained in logarithmic growth for 10 days to allow phenotypic expression of induced mutants. Each independent culture was then selected in 40 μM 6-TG (Sigma), and 6-TG^r clones were isolated as described previously (17). Independent induced mutants were defined as either those arising in different initial 10-cm dishes or unique mutants arising from the same initial culture, following DNA sequencing of *HPRT* cDNA.

PhIP-induced Mutant Frequency. A parallel induced mutant frequency experiment was performed with the same cells and PhIP-containing medium to verify that mutants were induced by PhIP. The procedure and the induced mutagenic response at the *HPRT* locus to 10 μM PhIP have been reported previously (14). Briefly, HAT-cleansed cultures plated in triplicate (1.5×10^6 cells per 175-cm^2 flask) were exposed to the same PhIP-containing medium, allowed an equivalent 10-day expression period, and selected in 40 μM 6-TG as described. Nonexposed cultures were maintained in parallel as controls. 6-TG^r colonies were then fixed and stained, and *HPRT* mutant frequencies were calculated.

***HPRT* cDNA Amplification and Sequencing.** Amplification of *HPRT* mRNA from PhIP-induced mutant clones was performed as described (18). *HPRT* cDNA was directly amplified from cells by reverse transcription of *HPRT* mRNA, followed by amplification of the cDNA by nested PCR. The full-length cDNA product was then purified, and the molecular defect was determined by automated sequencing of the cDNA.

RESULTS

Prior to isolating independent *HPRT* mutants induced by PhIP, a parallel induced mutant frequency experiment was performed with the same PhIP-containing medium used to induce independent cultures (see "Materials and Methods"). The results of these experiments are presented in Fig. 1. In the DLD-1 cell line, the spontaneous *HPRT* mutant frequency was $85 \pm 14 \times 10^{-6}$, whereas the frequency in the PhIP-exposed cultures was $210 \pm 20 \times 10^{-6}$. This corresponded to a 2.5-fold increase in the number of mutants induced by PhIP exposure in DLD-1 cells. For HCT116, the spontaneous *HPRT* mutant frequency was $150 \pm 20 \times 10^{-6}$, whereas the frequency of mutants in the treated cultures was $320 \pm 35 \times 10^{-6}$. Here, a 2.1-fold induction of mutants was observed after PhIP treatment. Because the PhIP-containing medium used to generate these data were the same medium used to treat individual cultures, it can be concluded that the independent mutants isolated from individual PhIP-exposed cultures did in fact contain mutations that were induced by PhIP. On average, this 2.3-fold induction of mutants implies that 56% of all mutants collected after PhIP exposure will be true PhIP-induced mutations.

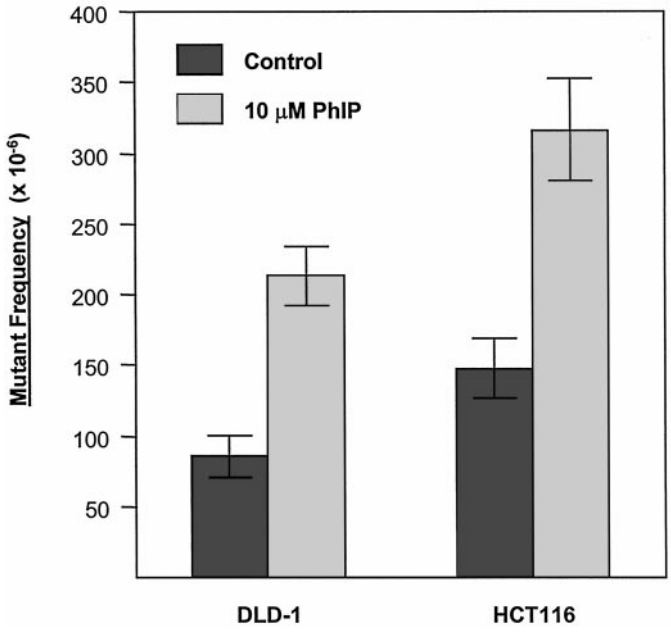

Fig. 1. Mutant frequencies at the *HPRT* locus in the DLD-1 and HCT116 cell lines for untreated (control) and PhIP-induced (10 μM) cultures. Mutant frequencies presented are an average of three determinations; *bars*, SD.

Table 1 *Summary of spontaneous and PhIP-induced mutations at the HPRT locus in the HCT116 and DLD-1 cell lines*[a]

	DLD-1	DLD-1 + PhlP	HCT116	HCT116 + PhlP
Base substitutions[b]	35 (88)	42 (95)	31 (69)	32 (76)
Transitions				
G:C → A:T	14 (35)	17 (39)	21 (47)	15 (36)
A:T → G:C	5 (13)	1 (2)	2 (4)	3 (7)
Transversions				
G:C → C:G	0	0	0	1 (2)
G:C → T:A	6 (15)	12 (27)	3 (7)	7 (17)
A:T → C:G	0	2 (5)	0	1 (2)
A:T → T:A	0	2 (5)	1 (2)	2 (5)
Putative splice[c]	10 (25)	8 (18)	4 (9)	3 (7)
Frameshifts	5 (13)	2 (5)	14 (31)	10 (24)
Total number of mutants	40 (100)	44 (100)	45 (100)	42 (100)
Mutations in run of Gs[d]	1 (3)	10 (23)	9 (20)	13 (31)
Transitions	0	4 (9)	0	3 (7)
Tranversions	1 (3)	4 (9)	0	2 (5)
+1 G	0	2 (5)	9 (20)	6 (14)
−1 G	0	0	0	2 (5)

[a] Incidence (percentage of total); spontaneous spectra reported previously (17).
[b] Includes putative splice mutations as base substitutions.
[c] Either exon deletions or deletion/insertion of several bases at exon junctions.
[d] Run of six guanines, base 207 to 212 in exon 3.

Table 2 *Relative mutant frequency per type of mutation for spontaneous and PhIP-induced mutations in the DLD-1 cell line*

	Spontaneous mutant frequency[a] ($\times 10^{-6}$)	PhlP-induced mutant frequency[a] ($\times 10^{-6}$)	Fold increase[b]
Total mutant frequency[c]	85	210	2.5
Base substitutions	74	200	2.7
Transitions	40	86	2.2
Transversions	13	76	5.8
Frameshifts	11	10	0.9
Mutations in run of Gs[d]	2	48	24

[a] Total mutant frequency multiplied by the incidence of each type of mutation presented in Table 1.
[b] Fold increase induced by PhlP relative to spontaneous mutant frequency.
[c] Total mutant frequencies determined in a parallel induced mutant frequency experiment.
[d] Run of six guanines, base 207 to 212 in exon 3; includes all types of mutations as listed in Table 1.

The mutational spectrum induced by PhIP at the *HPRT* locus for *hMSH6*-defective DLD-1 cells was obtained from 44 independent mutants. The resulting spectrum is presented in Table 1. This spectrum was predominately base substitution mutations (95%), with the majority being transitions (Table 1). The incidence of transition mutations was approximately the same in the spontaneous spectrum and the PhIP-induced spectrum (48% *versus* 41%, respectively; Table 1). When considering transversion mutations, however, a statistically significant increase in the incidence was observed between spontaneous and PhIP-induced mutations (15% *versus* 37%, respectively; $P < 0.05$, two-tailed Fisher's exact test).

To calculate the relative mutant frequencies of particular mutation events, the incidence of a particular mutational event is multiplied by the total mutant frequency in the culture. These values are presented in Table 2 for the DLD-1 cell line. From these values, a significant 2.7-fold increase in the frequencies of base substitutions is observed in the PhIP-treated cultures. Transversion mutations account for a greater contribution to this increase than transition mutations. With transversion mutations, a 5.8-fold increase in the frequency of muta-

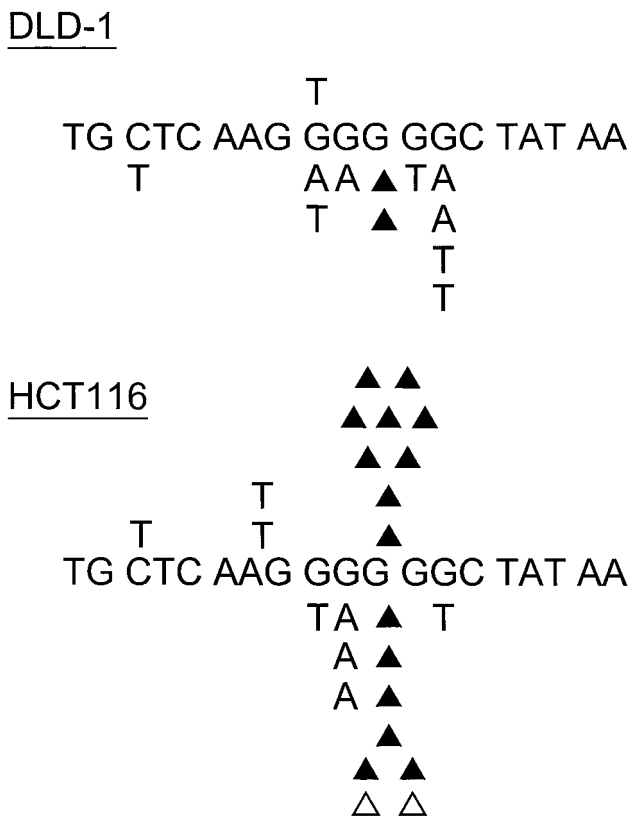

Fig. 2. PhIP-induced hotspot mutations within the run of six guanines in *HPRT* exon 3, base 207–212. The primary wild-type sequence is shown. Spontaneous mutations observed are illustrated *above* the sequence, whereas PhIP-induced mutations are *below* the sequence. For frameshift mutations: △, loss of a guanine; ▲, addition of a guanine. For HCT116 spontaneous mutations, there were two +T insertions at base 207. See Table 1 for the total number of mutations sequenced from each group.

tion relative to spontaneous is observed with PhIP, whereas transition mutations show a 2.2-fold increase.

When considering the location of the PhIP-induced mutations within the coding region of the *HPRT* gene, we see marked predisposition for PhIP-induced mutation within a run of six guanines in exon 3 at base 207 to 212. These mutations consisted of a variety of mutational events, such as frameshifts and transition and transversion mutations, and are at almost every position within the run (Fig. 2). Spontaneously, only one mutant of 40 (3%) was seen in this homopolymeric run (G to T transversion at base 208), whereas PhIP induced 10 mutants of 44 (23%) in the same location (Table 1 and Fig. 2). As mentioned above, only 56% of the 44 mutants collected in the PhIP-induced cultures are expected to be induced by PhIP (because of elevated spontaneous mutant frequency), or ~24 independent PhIP-induced mutants. Thus, approximately half (10 of 24) of actual PhIP-induced mutants occurred within this homopolymeric run. A substantial 24-fold increase in the mutant frequency over the spontaneous frequency was observed in this run (Table 2). It is interesting to note that two of the PhIP-induced mutations observed within the homopolymeric run were +G frameshift mutations (Table 1). Because spontaneous frameshift mutations were not observed within this homopolymeric run, it can be concluded that PhIP exposure was responsible for these frameshift mutations.

For the *hMLH1*-defective cell line, HCT116, the PhIP-induced mutational spectrum was similar to that seen in DLD-1. The spectrum was generated from 42 independent PhIP-induced mutants and was predominately base substitution mutations (76%), with the majority being transitions (Table 1). For transition mutations, the incidence seen with PhIP treatment (43%; Table 1) is similar to that seen spontaneously (51%; Table 1). This corresponds to a 1.8-fold increase in the specific frequency of PhIP-induced transition mutation relative to spontaneous frequencies (Table 3). With transversions, however, a statistically significant increase in the incidence was observed between PhIP-induced and spontaneous mutations (26% *versus* 9%, respectively; $P < 0.05$, two-tailed Fisher's exact test). This corresponded to a significant 3.1-fold increase in relative mutant frequencies for transversion mutations in HCT116 cells.

Table 3 *Relative mutant frequency per type of mutation for spontaneous and PhIP-induced mutations in the HCT116 cell line*

	Spontaneous mutant frequency[a] ($\times 10^{-6}$)	PhlP-induced mutant frequency[a] ($\times 10^{-6}$)	Fold increase[b]
Total mutant frequency[c]	150	320	2.1
Base substitutions	100	240	2.4
Transitions	77	140	1.8
Transversions	27	84	3.1
Frameshifts	46	76	1.7
Mutations in run of Gs[d]	30	99	3.3

[a] Total mutant frequency multiplied by the incidence of each type of mutation presented in Table 1.
[b] Fold increase induced by PhlP relative to spontaneous mutant frequency.
[c] Total mutant frequency determined in a parallel induced mutant frequency experiment.
[d] Run of six guanines, base 207 to 212 in exon 3; includes all types of mutations as listed in Table 1.

PhIP-induced mutations were observed throughout the coding region of the *HPRT* gene in the collection of 42 independent mutants from HCT116. We did, however, observe a cluster of mutations within the homopolymeric run of guanines in exon 3. Thirteen of the 42 PhIP-induced independent mutations, or 31%, are seen within this run of six guanines, whereas 9 of 45, or 20%, are seen spontaneously (Table 1). This hotspot for mutation accounts for a third of all PhIP-induced mutations observed in HCT116. Only +G frameshifts are seen spontaneously within the run, whereas the PhIP-induced mutations within this run now include −G frameshifts and transition and transversion mutations (Fig. 2). The relative mutant frequency for PhIP-induced mutations within this run of guanines (Table 3) is 3.3-fold higher than in the spontaneous spectrum. The fold increase is not as high as that seen with DLD-1 (24-fold elevation; Table 2), attributable to the significantly elevated spontaneous mutant frequency in HCT116 seen within this run. Even with an elevated background level of mutations within this run, the absolute increase in frequency was 69×10^{-6} (Table 3; PhIP-induced frequency minus spontaneous frequency). It can be concluded that PhIP-induced mutations tend to predominate within this homopolymeric run in HCT116 cells.

DISCUSSION

Here we present data on the specificity of mutations induced by the HA PhIP in cells that are defective in MMR. Recently, we described the cellular response to PhIP exposure in both the DLD-1 and HCT116 cell lines (14); these cell lines were more resistant to cytotoxicity and were hypermutable relative to MMR-proficient matched chromosome transfer lines. The illustration of a hypermutable response in both HCT116 and DLD-1 relative to their respective MMR-proficient chromosome transfer lines (14) suggests that the induced mutational specificity studied here is a direct result of PhIP exposure in the absence of MMR. The demonstration that MMR mediates the cellular response to PhIP exposure, a common food-associated mutagen/carcinogen, suggests that individuals compromised in MMR may

be more susceptible to colorectal carcinogenesis from dietary mutagens/carcinogens. Therefore, the particular types of mutations induced by PhIP in MMR-defective cells was determined to further elucidate the connection between MMR-mediated colorectal carcinogenesis and PhIP-induced colon carcinogenesis.

The major DNA adduct formed by PhIP is a bulky adduct at the C8 position of guanine (19), dG-C8-PhIP, which is the critical adduct in PhIP mutagenesis (19). Mutational specificity of PhIP-induced mutations in cell lines that are proficient in MMR illustrates a high percentage of G:C to T:A transversions (19–21), suggesting that dG-C8-PhIP directly miscodes with adenine. This dG-C8-PhIP:A mismatch may be the mismatch recognized by MMR, and in MMR-deficient cells, this induced mismatch may remain unrepaired. In the two MMR-deficient cell lines studies here, the predominant mutation observed was also G:C to T:A transversions (Table 1), consistent with PhIP inducing the adduct dG-C8-PhIP, the subsequent miscoding with adenine, and the lack of repair by MMR.

Additional mutations seen after PhIP exposure include frameshift mutations. Specifically, −G frameshift hotspots are seen in homopolymeric runs of guanine bases (20, 21) in human and rodent cells. One hotspot for −G frameshift mutations is the sequence GGGA (21), which has also been found in the *APC* tumor suppressor gene in five of eight colon tumors from PhIP-treated rats (22), suggesting that this mutagenic event may play a role in the observed carcinogenesis. Here PhIP-induced frameshift mutations were also seen in MMR-defective cells. In the HCT116 cell line, −G frameshifts were induced by PhIP within the homopolymeric run of six guanines of exon 3. Also, in the DLD-1 cell line, +G frameshifts induced by PhIP exposure were observed within this sequence. Together, these findings in both MMR-proficient and -deficient backgrounds implicate dG-C8-PhIP adducts in generation of frameshift mutations in homopolymeric sequences.

Supporting evidence for a role of MMR in PhIP-induced mutagenesis is provided from comparisons with another carcinogenic aromatic amine AAF. The mutational specificity of PhIP and AAF in MMR-proficient human and rodent cells suggests similar mutagenic mechanisms; the major AAF adducts are also bulky C8 guanine adducts, dG-C8-AAF or the deacetylated dG-C8-AF adduct, with a mutational specificity of primarily G:C to T:A transversions and frameshift mutations in homopolymeric runs (23, 24). More importantly, hMutSα (hMSH2/hMSH6) has been shown to bind to both the dG-C8-AAF or dG-C8-AF adduct (25), and MMR has been postulated to be involved in processing such damage. Considering the similarities in adduct structure and mutational specificity of dG-C8-PhIP and dG-C8-AAF, it can be hypothesized that MMR proteins are also involved in recognizing dG-C8-PhIP adducts.

To further investigate the putative link between the molecular mechanisms of carcinogenesis for MMR and PhIP, one can consider instability at microsatellite sequences. Instability at microsatellite sequences is a hallmark of MMR defects, and instability at these sequences has generally been used as a marker for MMR deficiencies (1–4). Several reports have demonstrated that PhIP-induced rat colon tumors exhibit microsatellite instability (26–29), suggesting a potential mechanism of tumorigenesis involving MMR. Given our recent findings that cells defective in MMR were more resistant to PhIP-induced cytotoxicity at the expense of hypermutability (14), perhaps PhIP is selecting cell populations that are MMR defective.

One other characteristic phenotype of MMR deficiencies is frameshift mutations in homopolymeric runs, including those in tumor suppressor genes (1–4). It is clear that certain key growth-regulating genes are mutated in MMR-defective tumors, suggesting a particular molecular mechanism for such tumors (30–34). These include mutations in the apoptosis gene *BAX*, the insulin-like growth factor II receptor *IGFIIR*, and even the mismatch repair gene *hMSH6*. These genes all contain homopolymeric runs of guanines in their coding regions that are preferentially mutated in tumors. As shown in this study in the DLD-1 and HCT116 cell lines, a hotspot for mutations induced by PhIP occurs at runs of guanines, including both −G and +G frameshift mutations. Perhaps PhIP plays a role in tumorigenesis in MMR-defective cell populations by promoting frameshifts within the homopolymeric runs of such genes.

Another interesting observation involves the β-catenin gene (*CTNNB1*), a proto-oncogene involved in the transcriptional regulation of other growth-regulating genes. Activating β-catenin mutations appear to be specific for human colorectal tumors that are defective in MMR (35). There is also an extremely high frequency of mutations in the β-catenin gene in rat colon tumors induced by PhIP (36), implicating these mutations in the molecular mechanism of PhIP-induced colon tumors. Because β-catenin mutations appear to be specific for MMR-defective colon tumors, perhaps PhIP is inducing tumors that are MMR deficient. Further investigation of the MMR status in these PhIP-induced tumors should provide insight into the putative link between PhIP and MMR deficiencies.

In summary, we have determined the mutational specificity of the food-associated HA, PhIP, in cells that are defective in MMR. We observed a significant increase in G:C to T:A transversion mutations in both cell lines, consistent with previous mutational spectra seen after PhIP exposure. More importantly, we see that a run of six guanines, base 207 to 212 in exon 3 of *HPRT*, is a hotspot for PhIP-induced mutation in MMR-defective cells in which frameshift mutations and base substitution mutations are induced. This suggests that homopolymeric runs of guanine bases may be hotspots for PhIP-induced mutation in MMR-defective cells. Interestingly, the molecular fingerprint in PhIP-induced tumors and MMR-defective tumors appears quite similar, suggesting a potential synergistic effect between PhIP exposure and MMR deficiency on the development of colon cancer.

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